REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
21-05-2010	Final Report	31-Jul-2006 - 1-Jun-2009

4. TITLE AND SUBTITLE
Final Report for "Human-based Polyclonal Antibodies to Ricin"

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W911NF-06-C-0070

5c. PROGRAM ELEMENT NUMBER
406038

6. AUTHORS
Thor Borgford

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
CANADIAN COMMERCIAL CORPORATION
8081 Lougheed Hwy

00000 -

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
50297-CH-CDP.1

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
The feasibility of producing a human polyclonal antibody to ricin intoxication was demonstrated. Investigators manufactured a relatively-benign yet full-length, ricin-like toxoid according to a cGMP standard -- with a view to immunizing healthy volunteers (plasma donors). The immunogenic potential of the toxoid was convincingly demonstrated in GLP and non-GLP animal studies and in a parallel Phase I human clinical trial (involving cancer patients). Simultaneously the post-exposure protective benefits of polyclonal antibodies where shown in rodents

15. SUBJECT TERMS
ricin, medical countermeasure, polyclonal, mononclonal, toxoid

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Thor Borgford
UU	UU	UU	UU		19b. TELEPHONE NUMBER: 778-999-7171

Report Title

Final Report for "Human-based Polyclonal Antibodies to Ricin"

ABSTRACT

The feasibility of producing a human polyclonal antibody to ricin intoxication was demonstrated. Investigators manufactured a relatively-benign yet full-length, ricin-like toxoid according to a cGMP standard -- with a view to immunizing healthy volunteers (plasma donors). The immunogenic potential of the toxoid was convincingly demonstrated in GLP and non-GLP animal studies and in a parallel Phase I human clinical trial (involving cancer patients). Simultaneously the post-exposure protective benefits of polyclonal antibodies where shown in rodents which were subjected to normally lethal doses of ricin. Despite the technical success, manufacture and stockpiling of a hyperimmune human polyclonal was ultimately not possible due to safety concerns related to the administration of ricin toxoid to healthy volunteers. In the concluding phase of the project, the investigators collected human peripheral lymphocytes from consenting cancer patients who had received >6 months of treatments (weekly injections) with toxoid. Investigators are positioned to clone a high affinity anti-ricin monclonal from 'affinity-maturated' antibody-genes.

List of papers submitted or published that acknowledge ARO support during this reporting period. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Number of Papers published in peer-reviewed journals: 0.00

(b) Papers published in non-peer-reviewed journals or in conference proceedings (N/A for none)

Number of Papers published in non peer-reviewed journals: 0.00

(c) Presentations

Borgford, T (June 2008) "Ricin Toxoids & Antibody-based Therapeutics" NIAID Ricin & Shiga Toxin Workshop, Albany, New York

Number of Presentations: 1.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts): 0

(d) Manuscripts

Number of Manuscripts: 0.00

Patents Submitted

Patents Awarded

Graduate Students

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Post Doctorates

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Faculty Supported

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT SUPPORTED	
Larry Gontovnick	0.13	No
Thor Borgford	0.10	No
Curtis Braun	0.21	No
Veronica Restelli	0.73	No
Carla Zimmerman	0.63	No
Admir Purac	0.35	No
FTE Equivalent:	**2.15**	
Total Number:	**6**	

Sub Contractors (DD882)

Inventions (DD882)

SECTION A: FORWARD

Broad Agency Announcement

Twinstrand Therapeutics Inc. (Twinstrand) responded to the Broad Agency Announcement # W911NF-05-R-0010 and was successfully funded (contract W911NF-06-C-0070) for the project described in this final report; **Human Based Polyclonal Antibodies to Ricin - Post Exposure Countermeasure**.

Notably the BAA sought, in part, *"Development and generation of high quality human-based polyclonal antibodies — for pretreatment or post-exposure prophylaxis against BW threats."* At the outset, Twinstrand had a decade of experience in the development of ricin-like molecules for medical/therapeutic purposes and experience in the development of non-human (equine, caprine, murine) polyclonal antibodies to ricin.

The Ricin Threat

The ricin threat derives from several factors;

- **i)** mole-for-mole, ricin is one of the most toxic substances known (1),
- **ii)** the toxin may be readily extracted from castor beans using low-tech methods which are accessible to relatively unskilled persons,
- **iii)** castor bean plants are widely cultivated throughout the world both as an oil crop an as an ornamental ,
- **iv)** as an agent of terror, purified ricin can be readily distributed, and highly toxic, in a powdered or aerosolized form, and
- **v)** there is currently no antidote to ricin poisoning adding to public anxiety and the potential impact of a ricin attack.

Summary of Results

During the course of the project, Twinstrand clearly demonstrated the technical feasibility of producing human hyperimmune polyclonals for ricin poisoning. The investigators developed a relatively-benign, ricin-like, recombinant known as TST10088 and successfully manufactured the toxoid according to cGMP standards. The immunogenic potential of the toxoid was demonstrated in GLP and non-GLP animal studies and in a human clinical trial (involving cancer patients). Further, the post-exposure protective benefits of polyclonal antibodies where demonstrated in rodents which were subjected to normally lethal doses of ricin.

Despite the technical success, manufacture and stockpiling of a hyperimmune human polyclonal for emergency use was halted by safety/regulatory concerns (FDA mandated) related to the administration of ricin toxoid to healthy volunteers. Nevertheless, in the concluding phase of the project, Twinstrand received ethics approval to collect human peripheral lymphocytes from consenting cancer patients in order to clone human antibody-producing genes. The investigators are now positioned to create high-affinity monoclonal antibodies that are not susceptible to the same safety concerns associated with the production of a human hyperimmune polyclonal.

Project Legacy

In July 2009, shortly after the official conclusion of the DTRA project, Twinstrand Therapeutics was acquired by Cangene Corporation, a company with considerable expertise in the development and commercialization of antibody countermeasures to biowarfare/terror agents. Cangene has core competence in the development, manufacture, clinical trial, sales and marketing of polyclonal and monoclonal products and is a major supplier of antibody-based countermeasures to the US Department of Health and Human Services (under project Bioshield). No other company is better situated to take advantage of the legacy of Twinstrand's DTRA-funded ricin countermeasure project.

SECTION B: TABLE OF CONTENTS

SECTION C: LIST OF ILLUSTRATIONS:

SECTION D: STATEMENT OF THE PROBLEM STUDIED:

Specific Project Goals

The overarching goal of the project was to create an antibody-based countermeasure for the treatment of ricin poisoning. Responding to the Broad Agency Announcement, the investigators elaborated a project to create a *"human polyclonal countermeasure to ricin"* that would provide 'passive' immunity to ricin. Precedent products of this nature had been developed elsewhere (2), several of which are potentially able to give pre-exposure and/or post-exposure protective benefits (3).

In brief, the project was designed to follow the steps;

1) Create and characterize a cGMP ricin toxoid suitable for the immunization of healthy volunteers.
2) File an Investigational new Drug Application (IND) for Phase I immunization with ricin toxoid.
3) Hyperimmunize healthy volunteers through a series of intramuscular injections (as vaccine).
4) Isolate/purify anti-ricin immunoglobulin from volunteers by plasmaphoresis.
5) File a second IND for the Phase I safety testing of the anti-ricin immunoglobulin.
6) Develop post-exposure protocols (using animal models) for the administration of protective immunoglobulin.

Additional objectives included;

7) Characterize the immune response and toxicity to a ricin-like toxoid.
8) Determine the effects of anti-inflammatory agents on antibody therapy/ ricin toxicity
9) Collect human peripheral lymphocytes from immunized subjects in order to clone monoclonal antibody genes.

Project Rationale

Why a polyclonal countermeasure?

A substantial body of literature exists (4) concerning the production and therapeutic use of hyperimmune immunoglobulins that suggested that a human immunoglobulin countermeasure could be generated relatively quickly (i.e., as a "stop gap" measure) using toxoid(s) that Twinstrand was already developing for the treatment of cancer. Ricin monoclonals and ricin vaccines require longer development times and appeared to have much more problematic development pathways.

Vaccines are extremely effective when used to treat infectious agents. However, for a vaccine to be effective in an 'acute' ricin attack, the timing of the event must be anticipated with a high degree of accuracy to permit the immunization or booster of at-risk personnel to insure sufficient levels of circulating/secreted immunoglobulin to neutralize ricin. For ethical/regulatory reasons, it may not be practical to immunize a large cross-section of at-risk personnel.

The development of a humanized monoclonal was considered by the investigators a long-term solution for the need for a ricin countermeasure. However, monoclonals may require multiple cycles of selection, characterization and scale-up that could add substantially to the development time.

Notably, polyclonals may be more effective than monoclonals at neutralizing ricin because they contain multiple immunoglobulin species (idiotypes) that recognize different antigenic determinants (epitopes) associated with a foreign toxin. This is relevant to the development of a ricin countermeasure because there are multiple cultivars of the castor bean plant that produce different ricins each with a different spectrum of epitopes. A polyclonal antibody preparation could be active towards ricins from different cultivars where a monoclonal selected for a unique epitope may not.

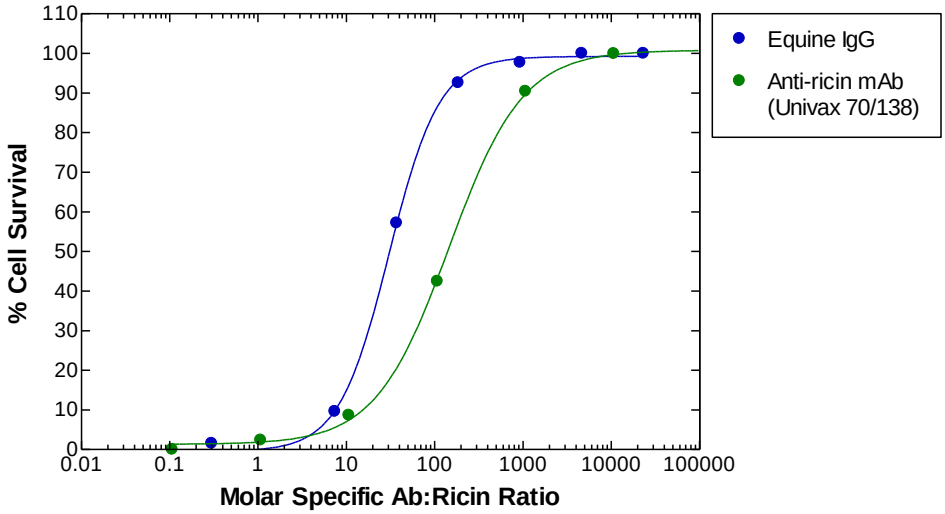


Figure 1: Protective benefits of polyclonal and monoclonal antibodies to ricin
The protective benefits of an equine polyclonal antibody (raised to TST10088) are compared to the relatively well-characterized Univax 70/138 monoclonal antibody in a standardized cytotoxicity assay. Antibody and ricin (i.e., ricin at a fixed concentration) were premixed and incubated and then 'fed' to Cos-1 cells in culture. Equine polyclonal antibody demonstrated a nearly 10 fold greater protective effect on the growth of COS-1 cells than the monoclonal antibody (i.e., at equivalent concentrations).

Why a "human" polyclonal?

Equine-derived polyclonals have been developed as snake anti-venoms and equine antibodies are currently in development (i.e., available for emergency use) for the treatment of botulinum toxin (5) and anthrax toxin (6). Non-human immunoglobulins are, however, prone to a common and potentially life-threatening adverse reaction known as "serum sickness" (7). Serum sickness is not typically associated with human polyclonal products.

Rationale for post-exposure therapy with a polyclonal antibody?

The relatively 'slow' pharmacokinetics of inhaled or ingested ricin creates a window of opportunity to ameliorate or neutralize toxicity with a medical countermeasure. Twinstrand envisioned post-exposure administration of a human polyclonal immunoglobulin via either intravenous or inhalation routes before the ricin toxin has time to enter the circulation or cause irreparable lung damage (in the case of an aerosol attack).

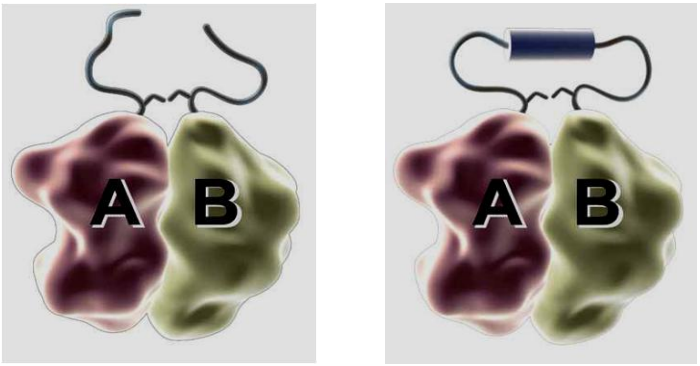
Advantages of Twinstrand Technology?

Prior to this start of this project Twinstrand Therapeutics had invested a decade in the development of ricin-like molecules for therapeutic purposes and had constructed several recombinant ricin-like antigens. During 2005, Twinstrand initiated a Phase I Human (safety) trial of the ricin-like TST10088 in cancer patients in the United States and Canada. Also, Twinstrand had undertaken preliminary studies to show that TST10088 could be safety administered to horses by intramuscular injection (i.m.) and elicit a significant antibody titres in that animal species.

SECTION E: SUMMARY OF THE MOST IMPORTANT RESULTS:

TST10088: a full-length, GMP, ricin-like toxoid was created and manufactured.

Ricin is a 60 KDa glycoprotein comprised of an A-chain and B-chain (Figure 2) that are held together by a single disulfide linkage. A-chain is an N-Glycosidase enzyme that catalytically inactivates ribosomes in eukaryotic cells whereas the B-chain is a lectin which is required to convey the protein into the target cell by receptor mediated endocytosis. Once internalize, the A-, and B-chain are released from one another by reduction of the disulfide bond that holds the halves of the molecule together.



Ricin TST10088

Figure 2 Schematic representation of ricin and the toxoid TST10088.
TST10088 is a relatively benign version of the ricin toxin in which the A-chain and B-chain components are linked by both a disulfide bond and 8 amino acid peptide. The peptide linkage contains a sequence of amino acids that is the substrate for metalloproteinase enzymes. TST10088 is not toxic so ong as the 2 chains of the molecule stay together.

Pro-ricin is the precursor form of ricin in which the two domains of the protein are held together by both a disulfide and a 23 amino acid peptide linkage. During the normal maturation of proricin to ricin in the castor bean, the peptide linkage is cleaved by a castor bean encoded protease. Importantly, so long as the two domains of the protein remain linked (i.e., the A-chain cannot escape from the B-chain inside a target cell), proricin is many orders of magnitude less toxic that mature ricin. Using recombinant method, the investigators constructed and express in *Pichia* yeast, a variant of the proricin protein known at TST10088 (Figure1) which was roughly 1000 times less cytotoxic than the mature form of ricin (Figure 3).

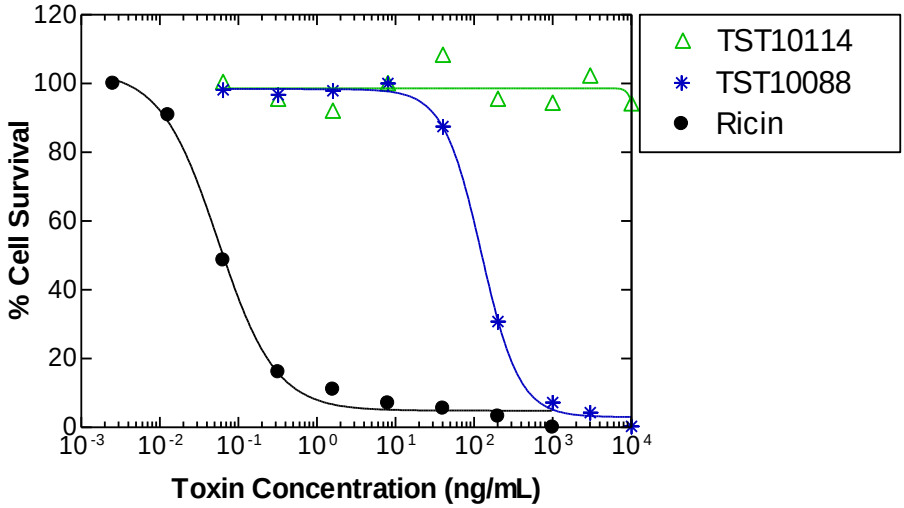


Figure 3: Relative cytotoxicity of ricin and TST10088 to COS-1 cells in culture.
The toxicity of ricin and recombinant variants of ricin are shown in a cell-based assay. The GMP antigen TST10088 is a relatively benign version of ricin in which the chains are linked with a short peptide. The non-GMP antigen known as TST10114 is nearly identical to TST10088 but carries an additional inactivating mutation in its A-chain.

Due to ricin's is extreme toxicity, it has been the practice to use inactivated or denatured forms of the protein to elicit an immune response in vertebrates. For example investigators elsewhere have used; the ricin A-chain alone (8), ricin fragments (9), or chemically denatured forms of ricin.

A central premise of the DTRA study described here was that the ricin-like TST10088, despite its severely attenuated toxicity, would elicit highly effective polyclonal antibodies. As compared to other immunogens, TST10088 retains native epitopes of both A-, and B-chains.

Initially, TST10088 protein was used to elicit high titre antibodies in rodents and horses. Anti-TST10088 antibodies were subsequently shown to cross-reacted to ricin with equal avidity (Figure 4). This observation suggested that TST10088 is an effective surrogate for ricin in animals.

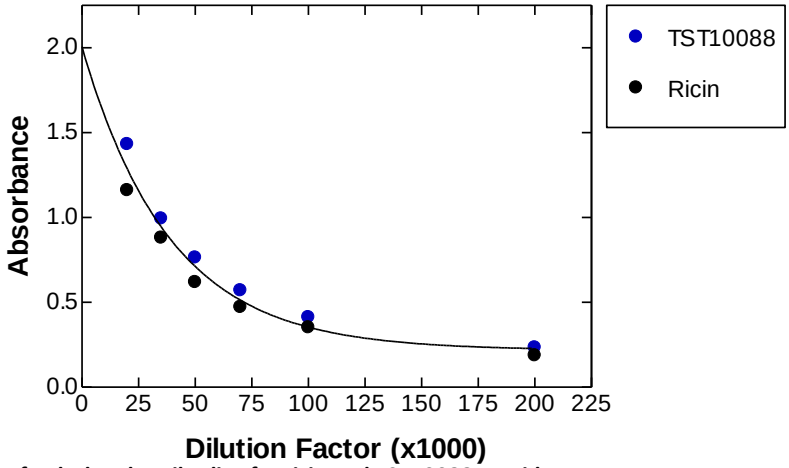


Figure 4: Avidity of polyclonal antibodies for ricin and TST10088 toxoid.

Polyclonal antibodies to TST10088 where raised in rodents. The ability of anti-sera to bind ricin or TST10088 was measured in a cell-free enzyme-linked immunosorbant assay (ELISA).

TST10088 administered to humans at 200 times the MTD of authentic ricin.

TST10088 was developed with a dual purpose in mind; 1) to function as an toxoid immunogen in eliciting anti-ricin antibodies in healthy volunteers (the subject of this DTRA project) and 2) TST10088 was anticipated to have an antitumor activity (Note: the protein`s mechanism of action as an anticancer agent is not discussed in this document).

The development of hyperimmune antibodies for the treatment of ricin poisoning and the clinical development of TST10088 as a cancer therapy took place concurrently starting in 2005. A Phase I Trial of TST10088 was begun in the US and Canada (under IND and CTA applications respectively). The study involved a dose-escalating intravenously administration of the protein to multiple cohorts of patients (Figure 5). Beginning with a dose of 10 μg/m2 of TST10088, the study reached a maximum tolerated dose (MTD) of 5000 μg/m2. By comparison, investigators at the Norwegian Radium Hospital (10) determined the MTD for authentic ricin to be roughly 23 μg/m2.

COHORT	DOSE mcg/m2												
	DOUBLING							MODIFIED FIBONACCI					
	10	20	40	80	160	320	640	1100	1600	2100	2800	3800	5000
1	4												
2		1											
3			1										
4				1									
5					1								
6						2							
7							1						
8								3					
9									3				
10										3			
11											3		
12												11	
13													3

Figure 5: Dose-escalating study of TST10088 in cancer patients.
Starting at a dose of 10 μg/m2, 13 cohorts of patients received TST10088 on a weekly schedule. The number of patients in each cohort is shown within the blue box. The highest dose administered was 5000 μg/m^2 (i.e., maximum tolerated dose). Patients typically received 2 months of treatment whereas some patients remained on study for periods > 1 year. Dose limiting toxicities included rigors, arthralgia, myalgia, and a spectrum of flu-like symptoms. TST10088 was considered "well-tolerated" at all doses up to 3800 μg/m^2.

Hence, the clinical trial in cancer showed that TST10088 could safely be administered to humans at a dose that was virtually 200 times the MTD of ricin. This observation was consistent with pre-clinical toxicology studies in rats and dogs that predicted a human MTD of 4000 μg/m2.

Different cultivars of the castor bean plant contain ricins with different toxicities.

Ricin from three different origins was used in the study. These included a SIgma Chemical Co standard purchased in 1995 and ricin preparations from Canadian and Indian cultivars of the castor bean (seeds provided by the Canadian Defense Research Establishment). The three ricins had different specific activities in cytotoxicity assays and animal experiments that could not be attributed to the 'quality' of the preparations as all three proteins were purified using a functional lactose-agarose affinity chromatography step.

Differences in cultivar toxicity are likely related to differences in the post-translational modification of the respective ricin proteins. Notably, in a separate study, the investigators observed differences in the ricin-related toxicities of recombinant forms of toxoid that had a different spectra of glycan attached (ricin is a glycoprotein).

An *in vivo* assay used to calibrate the neutralizing activity of different preparations of polyclonal immunoglobulin revealed marked differences in the toxicity of ricin from different castor bean sources. Briefly, immunoglobulin was combined with the equivalent of 5X LD50 of ricin (and the mixture injected intra-peritoneal (i.p.) into mice. The lowest effective dose (LED) was the ratio of immunoglobulin to ricin that rescued the animals from ricin toxicity.

For example, a molar ratio of 72:1 immunoglobulin to ricin was required to rescue animals from the toxic effects of the ricin derived from Indian Castor bean whereas the same antibody was effective against a domestic (Canadian cultivar) at a ratio of 18:1.

Antibodies provide significant post-exposure protective benefits in rodent studies.

Rodents were challenged by exposure to lethal intranasal doses of ricin (typically at 3 to 5 X the LD50). Cohorts were subsequently treated, at various times post-exposure, with polyclonal antibodies administered either i.v. or intranasally (i.n.).

For the purpose of establishing the appropriate therapeutic protocol – the investigators used an equine polyclonal antibody to ricin that was shown to have a similar *in vitro* neutralizing activity to human immunoglobulin but was much more abundant (available). Protective benefits were apparent whether or not the antibody was administered intranasally (I.N.) or intravenously (I.V.). Not surprisingly, the survival benefit diminished according to the time elapsed between ricin exposure and antibody treatment.

In the example shown in Figure 6, a post-exposure benefit was seen in mice out to 8 hours. It is worth noting that, in rodents, the half life of TST10088 delivered i.v. is less than 30 minutes. In humans the half life of TST10088 was determined to be 4 to 6 hours (data not shown). Ricin would have similar, if not identical, pharmacokinetics in rodents and humans. Therefore, the window of opportunity for treating humans is thought to be much longer than the 8 hours observed in rodents.

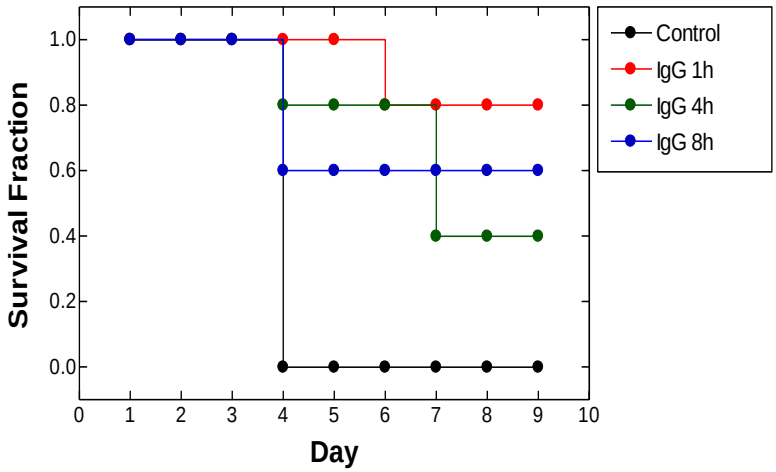


Figure 6: Post-exposure activity of equine polyclonal.

Rodents were exposed (intranasal) to 3 X LD50 of ricin. At intervals post-exposure, cohorts of animals were then administered polyclonal immunoglobulin by the intranasal route. Animal weighs and survival were monitored for a period of 10 days. A post-exposure benefit was evident to at least 8 hrs.

Dexamethazone augments the protective effects of antibodies in animal models.

Celebrex, a non-steroidal anti-inflammatory drug (NSAID), significantly ameliorated the infusion-related toxicities in humans (e.g., rigors, flu-like symptoms) at the highest does of TST10088 in humans in the clinical trial. Therefore, the investigators were encouraged to test anti-inflammatory combinations in animal studies where the challenge was natural ricin (not toxoid).

A study of the combination of the anti-inflammatory dexamethazone and polyclonal is shown in Figure 7. Notably, the combination reduced weight loss induced by ricin, delayed the onset of ricin toxicities and improved the survival of animals. Surprisingly, the effect of dexamethazone was insignificant when the drug was given on its own. Only in combination with immunoglobulin did animals benefit from the treatment.

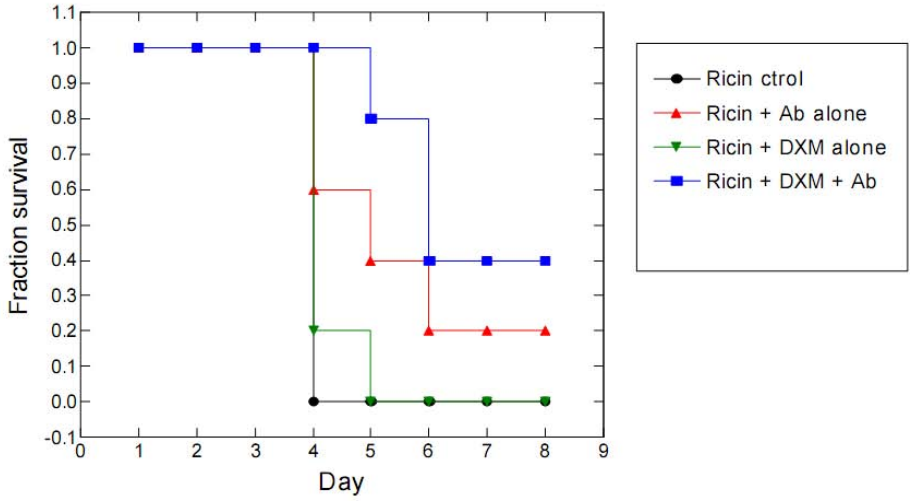


Figure 7: Survival data evaluating the benefits of the anti-inflammatory dexamethazone.
Rodents were given 3XLD50 intranasal exposure to ricin. Anti- inflammatory dexamethasone (DMX) was subsequently given 1 hr post-exposure and polyclonal antibody 8 hrs post-exposure. DMX produced a small but significant benefit in terms of weight loss and animal survival. DMX appeared to work synergistically with antiboy as there was no benefit from the drug on its own.

Alhydrogel did not enhance the immune response in dogs.

Alhydrogel (aluminum hydroxide) is an approved, commercially available and commonly-used human adjuvant. During formulation of porducts, protein antigen adhers to the solid-phase alhydrogel. The complexed material is then administered by injection.

Twinstrand experimented with several ratios of alhydrogel and toxoid in formulation studies and observed the immune response in both rats and dogs relative to the response in animals who received TST10088 alone (i.e., prepared in PBS). In preliminary rat studies, alhydrogel appeared to provide a boost to antibody titers. However, in subsequent dog studies there was no evidence that alhydrogel co-administered with toxoid improved titers.

Therefore, given that formulation with alhydrogel significantly increases the complexity and cost of the immunogen, the use of this adjuvant was not pursued. The lack of effect does not, of course, preclude the use of another adjuvant in future studies – as the benefits of a particular agent can only be determined empirically.

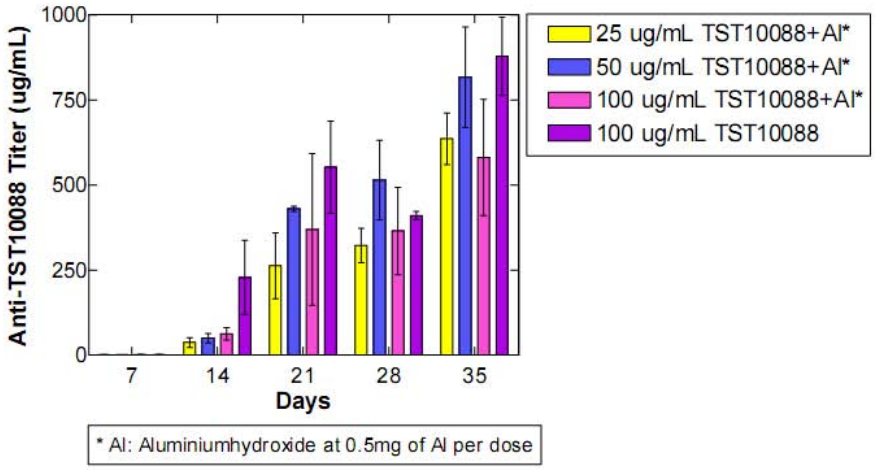


Figure 8: Effect of adjuvant on immune response

Dogs were immunized (i.m.) with varying amounts of the TST10088 toxoid bound to a fixed concentration of the common human adjuvant Aluminium hydroxide (Alhydrogel) or with TST10088 alone. At intervals following immunization, antibody titres were measured and immunoglobulin was harvested for further analysis. The use of adjuvant did not contribute to the antibody titres. In subsequent analysis, antibody drawn from animals that received no adjuvant appeared to have a greater neutralizing capacity than antibody derived from animals that received both TST10088 and adjuvant.

Human IgG contained relatively low-affinity antibodies after 8 weeks of innoculation

The TST10088 toxoid was administered to cancer patients in the Phase I study on a weekly basis and, similarly, antibody titers (to ricin and TST10088) were measured weekly. The majority of patients remained on study for a period of 8 weeks before the progression of their disease mandated a halt to treatment. Nevertheless, several patients experienced a benefit (e.g., stable disease or tumor regression) and remained on study for extended periods (e.g., 3 to 12 months). Figure 9 compares the antibody titers of 3 patients who received vastly different doses of TST10088.

Despite the range of doses, antibody titers were relatively low and insignificant within the 2 week period following the start of treatment. Titers reached a plateau within the first 2 months of treatment – remaining relatively low.

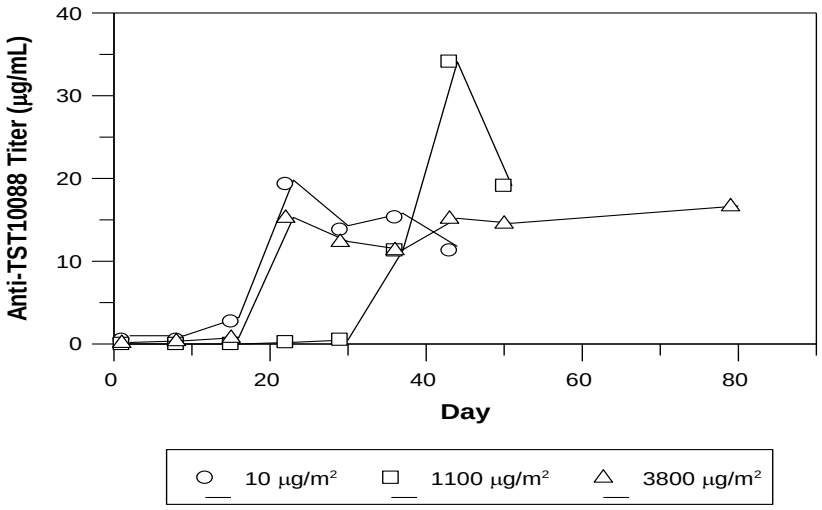


Figure 9: Human antibody titres do not appear to be dose dependant
Antibody titers were measured weekly in cancer patients treated with escalating doses of TST10088 between 10 µg/m² and 5000 µg/m² In the illustration titers are compared for dose levels 10, 1100 and 3800 µg/m² respectively. No significant differences in the absolute anti-TST10088 titers was observed (for this dose range) despite the vast differences in dose.

It is important to keep in mind that, in the studies shown in Figure 9, patients were 'immunized' with TST10088 by i.v. infusion as opposed to i.m. injection and the route of administration can produce diverse responses.

The threshold of safety proved too high to allow clinical studies in healthy volunteers.

Much of the rationale for the development of TST10088 as an immunogen came from the concurrent human safety studies where high doses of protein were given to cancer patients and TST10088 was categorized as "well-tolerated."

Cancer patients received the TST10088 protein by intravenous infusion whereas virtually all vaccines are administered by i.m. injection. In the studies proposed for healthy volunteers, intravenous infusion of TST10088 would be too invasive, too costly, technically impractical and unlikely to be acceptable to the FDA. Moreover, the immune response should be superior in patients who receive TST10088 by an i.m. route because protein is given greater exposure to dendritic (immune) cells in the skin (11). Therefore, the investigators proposed to deliver TST10088 toxoid by i.m. injection after completing preclinical toxicology studies in dogs.

Despite the successful i.v.administration of TST10088 to cancer patients and studies that showed TST10088 could be safely administered to horses by i.m. injection, the product was deemed to toxic to permit its use in health volunteers. At the highest doses used in pre-clinical toxicology studies, dogs experienced injection site reactions that included tissue necrosis. Lowering the i.m. dose ameliorated the local reaction and a safe starting dose was estimated for the studies proposed for healthy volunteers. However, the antibody titres of the i.m. – administered immunogen fell off with reduced dose and were not sufficient to warrant plasmaphoresis and immunoglobulin purification.

The threshold for the safety of immunogens/vaccines is very high in healthy volunteers who, unlike cancer patients, will not receive a direct clinical benefit from treatment. The investigators were unable to reconcile the need to administer high doses of TST10088 to elicit a significant immune response with the regulatory concerns for human safety.

Human antibody avidity for ricin increased significantly over time.

The immune response was elaborated in a single cancer patient who was treated weekly for roughly 6 months at a dose of 3800 μg/m^2. As in the example shown in Figure 9, antibody titers reached a plateau within 2 months (Figure 10a). However, the specific affinity of the polyclonal immunoglobulin drawn from the patient increased significantly and progressively (Figure 10b). The patient was eventually removed from study due to disease progression. The change in avidity presumably resulted from an affinity maturation of the antibody with repeated/continued exposure to TST10088.

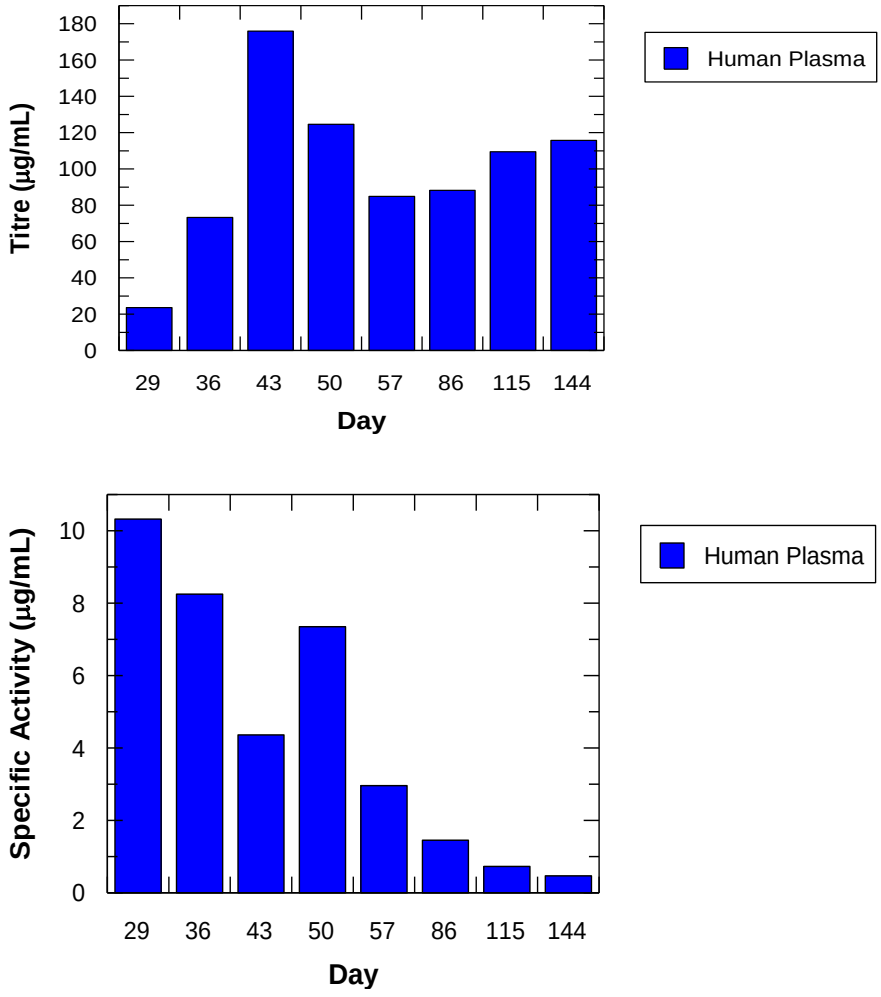


Figure 10: Antibody avidity increases with prolonged exposure to TST10088
The antibody titer (upper panel) and antibody specific activity (lower panel) are compared in an individual patient treated on a weekly schedule (with 3800 μg/m^2) over the course of 6 months. Although antibody titers are stabilized after 2 months of treatment, avidity improved progressively during the 6 months this patient was on study.

Human peripheral lymphocytes permit the cloning of anti-ricin monoclonal antibodies.

The observation that, over time, antibody 'avidity' improved suggested that high-affinity monoclonals could be cloned from human subjects who received multiple cycles of treatment with TST10088 (Figure 11).

The investigator sought and received ethics approval from the clinical sites (University of Colorado Cancer Center and BC Cancer Agency) to collect blood samples containing human peripheral lymphocytes. Informed consent was received from cancer patients already on study and two additional patients were recruited at the University of Colorado. The additional patients were brought on to compensate for the high attrition of subjects –improving the odds of at least one 'consented' patient continuing for 6 months of treatment while blood was collected. Blood was eventually collected from patients at both clinical sites and fractionated using a lymphoprep procedure. As a surprising bonus, the two new patients at the University of Colorado remained on study for a period of over 12 months.

Lymphocyte collection and cloning strategies were developed in conjunction with Dr. Jody Berry at the National Microbiology Lab in Winnipeg. Coincidentally, Dr. Berry was seconded to Cangene Corporation in 2009 – just prior to Twinstrand's acquisition by Cangene.

Cangene is now well positioned to complete the development and commercialization of an antiricin monoclonal product albeit beyond the timeline of this DTRA-funded project.

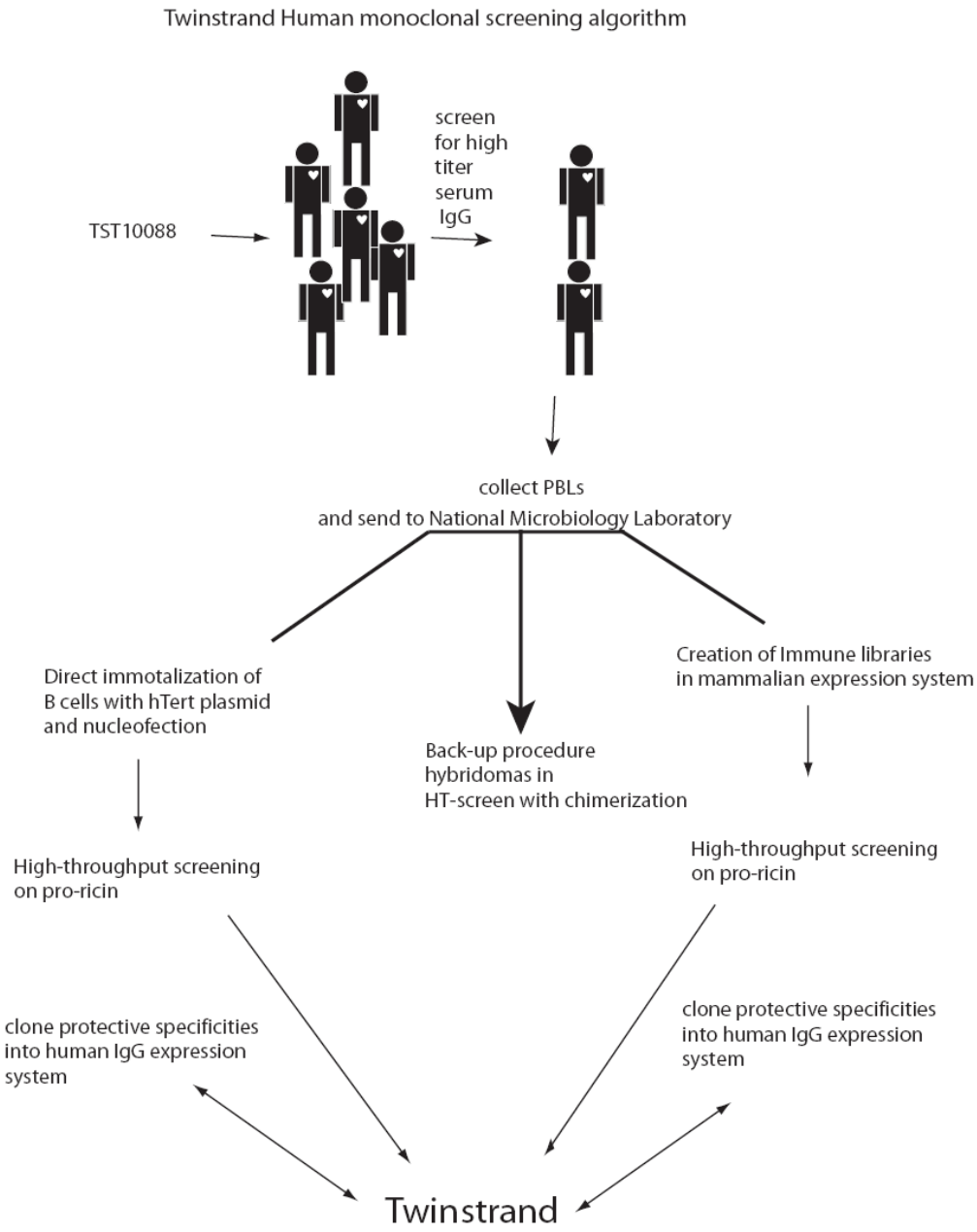


Figure 11 : Collection and cloning strategies for human monoclonals.
Several different strategies for lymphocyte collection and cloning were developed in collaboration with Dr. Jody Berry of the National Microbiology Laboratory. Ultimately, the path shown on the extreme right of the illustration was chosen.

Conclusions.

The project clearly demonstrated the technical feasibility of developing a human polyclonal countermeasure to ricin using the TST10088 toxoid (immunogen). TST10088 is a GMP-grade protein which received FDA approval for use in a Phase I clinical trial. During the clinical trial, TST10088 was administered to humans over a broad range of doses and was considered well-tolerated. The protein elicited an immune response and the neutralizing quality of the antiserum improved with repeated cycles of treatment. Pilot protocols in animals showed that immunoglobulin was capable of neutralizing ricin at safe/manageable doses.

Despite a successful technical conclusion, it was not possible to administer TST10088 to healthy volunteers to elicit a hyperimmune antisera to enable polyclonal manufacture. The local toxicity of the protein was unacceptable in healthy volunteers who would receive no medical benefit from treatment. This was a surprising and wholly unanticipated outcome to the project given the large number of patients who had been treated in the Phase I cancer trial and given the relatively low toxicity of TST10088 in horses who received local injections of the toxoid.

The investigators point to the need for a 'gold standard' animal model for testing and comparing ricin countermeasures which might include chemical agents, antibodies and vaccines.

Finally, in any future project to develop an antiricin polyclonal the investigators plan to use a totally benign toxoid (i.e., no ricin toxicity) derived from TST10088. For example, the investigators created the toxoid TST10114 (Figure 3) that is identical in structure to TST10088 but contains an additional inactivating amino acid substitution in the A-chain active site.

SECTION F: BIBLIOGRAPHY

1. Audi, J., Belson, M., Patel, M., Schier, J., & Ostertloh, J., (**2005**) *Ricin poisoning* J. Amer. Med. Assoc. 294: 2341-2351

2. Merck & Co (**2010**) *Immune globulins and antitoxins available in the US*. http://www.merck.com/mmpe/sec14/ch169/ch169c.html

3. Bhakdi, S., Mannhardt, U., Muhly, M., Hugi, F., Ronneberger, H., and Hungerer, K-D. (**1989**) *Human hyperimmune globulin protects against the cytotoxic action of staphylococcal alpha-toxin in vitro and in vivo.* Infection & Immunity 3214-3220

4. Bregenholt, S., & Haurum, J., (**2004**) *Pathogen-specific recombinant human polyclonal antibodies: biodefense applications*. Expert Opinion on Biological Therapy. 4: 387-396

5. National Institute of Allergy and Infectious Diseases. (**2002**) *Summary of the NIAID expert panel on botulinum toxins*. http://www.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/Documents/bot_toxins.pdf

6. Schneemann , A., & Manchester, M., (**2009**) Anti-toxin Antibodies in Prophylaxis and Treatment of Inhalation Anthrax. Future Microbiology. 4(1):35-43

7. Naguwa, S.M., & Nelson, B.L., (**2008**) *Human serum sickness.* Clinical Reviews in Allergy and Immunology 3: 117-126

8. Olson, M.A., Carra, J.H., Roxas-Duncan, V., Wannemacher, R.W., Smith, L.A., & Millard, C.B., (**2004**) *Finding a new vaccine in the ricin protein fold* Protein Engineering Design and Selection 17: 391-397

9. Vitetta, E.S., Smallshaw, J.E., Coleman, E., Jafri, H., Foster, C., Munford, R., & Schindler, J., (**2006**) *A pilot clinical trial of a recombinant ricin vaccine in normal humans*. Proc. Natl. Acad. Sci. 103:2268-2273

10. Fodstad, O., Kvalheim, G., Godal, A., Lotsberg, J., Aamdal, S., Host, H., & Pihl, A., (**1984**) *Phase I study of the plant protein ricin* Cancer Research 44: 862-865

11. Banchereau, J., & Steinman, R.M., (**1998**) *Dendritic cells and the control of immunity* Nature 392: 245-247